Approaches to drug resistance in solid tumors

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Chapter 4

Mechanisms for high methoxymorpholino doxorubicin cytotoxicity in doxorubicin-resistant tumor cell lines

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Abstract

Methoxymorpholino doxorubicin (MMRDX) is an anthracycline analog that is able to overcome tumor cell resistance to classical anthracyclines. Mechanisms for increased MMRDX cytotoxicity were analyzed in a small cell lung carcinoma cell line (GLC_4), its 300-fold doxorubicin resistant and multidrug resistance-associated protein (MRP) overexpressing subline (GLC_4/ADR), an ovarian carcinoma cell line (A2780) and its 100-fold doxorubicin resistant, P-glycoprotein overexpressing subline A2780AD.
Cross resistance, measured with MTT assay at MMRDX concentration resulting in 50% growth inhibition, was 1.8-fold in GLC_4/ADR and 4.5-fold in A2780AD compared to their respective parental cell lines. Cellular MMRDX accumulation was equal in GLC_4 and GLC_4/ADR and 2-fold lower in A2780AD compared to A2780.
Doxorubicin fluorescence was analyzed with confocal laser scan microscopy. Fluorescence was nuclear in sensitive, and cytoplasmic in resistant cell lines, while MMRDX fluorescence was found in the nucleus in all cell lines. Pre-incubation with the MRP blocker MK 571 restored in GLC_4/ADR cells the nuclear doxorubicin fluorescence pattern as observed in GLC_4 cells.
In conclusion, MMRDX can largely overcome cross-resistance in these P-glycoprotein and MRP overexpressing doxorubicin-resistant cell lines. Results suggest that MMRDX is no substrate for MRP mediated resistance.

Introduction

The clinical value of anthracyclines is limited by their toxicity profile and by intrinsic or acquired tumor drug resistance. Various mechanisms may play a role in tumor cell resistance to anthracyclines, including overexpression of drug efflux pumps, decreased levels of the target enzyme topoisomerase II and an increase in cellular detoxifying capacity. These drug efflux pumps, such as P-glycoprotein and MRP, are involved in ATP-dependent efflux of natural product drugs, resulting in decreased intracellular levels. Cellular anthracycline levels, however, do not always correlate with cytotoxicity and P-glycoprotein or MRP expression [1-3]. An explanation can be found in intracellular distribution of the drug, such as a shift from nucleus to cytoplasm or intracellular compartmentalization, both resulting in decreased cytotoxicity. Recently, intracellular vesicle transport of doxorubicin was shown for MRP overexpressing cells [4]. In resistant cell lines, the drug can

be localized in vesicles in the cytoplasm [1, 4]. This may explain the smaller differences between sensitive and resistant cell lines in cellular anthracycline accumulation compared to differences in resistance factor [2, 3].

The morpholino anthracyclines have been developed in the course of research aimed at identifying new anthracyclines with at least partially novel modes of action in addition to activity against P-glycoprotein and non-P-glycoprotein resistant tumors. Compared with other anthracyclines, morpholino anthracyclines are potent inhibitors of ribosomal gene transcription and, in contrast to other anthracyclines, cause topoisomerase I mediated DNA strand damage [5].
In vitro, morpholino anthracyclines show no cross resistance in doxorubicin resistant P-glycoprotein-positive and MRP-positive cell lines, cell lines with an altered topoisomerase II enzyme and cell lines resistant to cisplatin and melphalan. MMRDX is a morpholino anthracycline that has recently been enrolled into clinical trials.
MMRDX possesses a morpholino ring incorporating the amino nitrogen at the 3'-position of the daunosamine unit of the anthracycline molecule (figure 1). This modification of the molecule increases lipophilicity, cellular influx rate and consequently intracellular levels.
The aim of this study was to analyze and correlate cellular MMRDX levels and intracellular localization of MMRDX with MMRDX cytotoxicity in sensitive cell lines and their P-glycoprotein or MRP overexpressing doxorubicin resistant sublines. In addition, the role of the MRP blocker MK 571 on

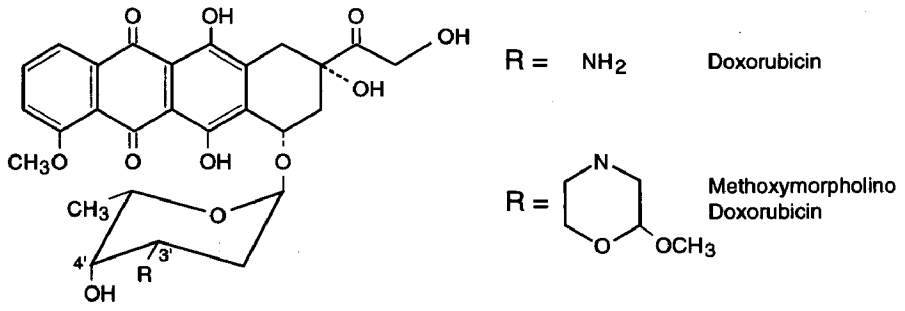


Figure 1 *Molecular structures of doxorubicin and MMRDX*

cellular doxorubicin and MMRDX distribution was studied in the sensitive and MRP overexpressing small cell lung cancer cell line.

Materials and methods

Chemicals

MMRDX and daunorubicin were gifts, and doxorubicin was purchased from Pharmacia (Milan, Italy). Roswell Park Memorial Institute (RPMI) 1640 medium, fetal calf serum (FCS), Hanks' balanced salt solution, Dulbecco's modified eagle (DME) and Ham's F12 media were purchased from Life Technologies (Paisley, UK). Dimethyl sulfoxide was purchased from Merck (Darmstadt, Germany), and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide) was obtained from Sigma (St Louis, MO). MK 571 was kindly provided by Dr. Ford-Hutchinson, Merck Sharp, Canada.

Cell lines

The cell lines used were GLC_4, a human small cell lung carcinoma cell line and its 300-fold doxorubicin resistant subline GLC_4/ADR. Resistance in GLC_4/ADR is in part due to overexpression of MRP, and a 65% reduced topoisomerase II activity [6, 7]. A2780 is a doxorubicin sensitive, and A2780AD its 100-fold doxorubicin resistant P-glycoprotein overexpressing ovarian carcinoma cell line [8]. There was no difference in cell size between the sensitive cell lines and their sublines. Previously, in GLC_4/ADR a 2.2-fold and in A2780AD a 2.9-fold lower cellular doxorubicin level than in the sensitive lines were observed [9, 10]. In order to assure stable resistance, GLC_4/ADR and A2780AD cells were cultured with respectively 1.2 and 2.0 μM doxorubicin twice weekly. Before the start of the experiments cells were cultured in drug-free medium for 3 weeks.

The microculture tetrazolium (MTT) assay

The MTT assay is based on the cellular reduction of the tetrazolium bromide MTT in viable cells to a blue formazan product that can be measured spectrophotometrically. Before the assay was performed the linear relationship of viable cells and formazan crystal formation was checked and cell growth studies were performed. For GLC_4 3750 cells, for GLC_4/ADR 10000 cells, for A2780 1250 and for A7280 AD 5000 cells per well in logarithmic phase of growth were incubated in 0.1 ml RPMI 1640 supplemented with 10% FCS in 96-well microtiter plates (Nunc, Life Technologies, Paisley, UK), at

37°C in a humidified atmosphere with 5% CO_2, with increasing concentrations of MMRDX.
At day 4, 20 µl of MTT solution (5 mg MTT/ml phosphate bufferred saline (PBS)) was added. After 3.75 h the plates were centrifugated (30 min, 180 *g*), supernatant was aspirated and 200 µl 100% dimethyl sulfoxide was added to dissolve formazan crystals. Extinction was read at 520 nm by scanning microtiterwell spectrophotometer (Titertek Multiscan, Flow Laboratories, Irvine, UK). The surviving fraction was calculated by the ratio of mean extinction of test sample to mean extinction of untreated sample. Controls consisted of media without cells (background extinction) and cells in medium without the drug. The mean concentration that caused 50% cell kill (IC_{50}) was determined in three independent experiments each performed in quadruplicate.

Cellular MMRDX levels
Samples containing 2-3 x 10^6 cells in RPMI 1640 medium with 10% FCS were incubated for 1 h with 100 µl of 0, 8, 16, 39, 78 or 1500 nM MMRDX at 37°C in a humidified atmosphere with 5% CO_2. Dose levels (7.8-78 nM) were based on peak plasma pharmacokinetic data from patients treated with MMRDX 1.5 mg/m^2 intravenous bolus [11]; 1.5 µM was added to include MMRDX IC_{50} values of the studied cell lines. Control samples were placed on ice, and to these samples MMRDX was added after 1 h, just before all samples were centrifuged at 180 *g*, 4°C. Cells were washed twice with phosphate bufferred saline (PBS, 0.14 M NaCl, 2.7 mM KCl, 6.4 mM $Na_2HPO_4.2H_2O$, 1.5 mM KH_2PO_4) at 0°C. After the second washing, cells were resuspended in 5 ml PBS, counted, centrifuged, pipetted and stored in polypropylene tubes at -20°C until analysis.
Cellular MMRDX levels were analyzed with a novel method using high-performance liquid chromatography and fluorescence detection. The detection limit for MMRDX in cells was found to be 0.75 nM. For the validation of MMRDX and its 13-dihydroxy metabolite (FCE 26176, 13-dihydro-3'-deamino-3'-[2(S)-methoxy-4-morpholino] doxorubicin) the method of Breda et al. was used [12]. After thawing, the cell pellet was resuspended in 1 ml PBS and 100 µl 0.038 mM daunorubicin as internal standard was added. Cells were buffered with 2.0 mL 0.5 M borate buffer saturated with NaCl (pH 8.4). After addition of 4 mL diethylether:*n*-butanol mixture (9:1, v/v) the solution was exposed to ultrasonic vibration for 10 min and 3 min centrifuged at 1600 *g* at 20°C. After 3 min at -53°C the upper organic layer was transferred to a silanized glass tube and the extraction procedure was repea-

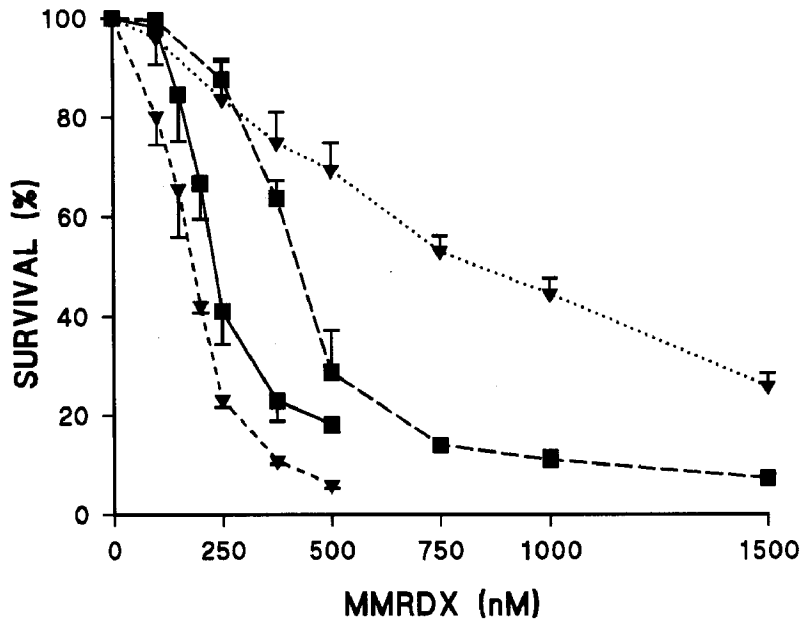
ted. Phosphoric acid (500 µl 0.05 M) was added to the combined organic phases. This solution was again vortexed 1 min, centrifuged 3 min at 1600 *g* and placed 3 min at -53 °C. The organic phase was discarded and the acidic phase was washed with 1 ml *n*-hexane by vortexing 1 min, centrifuged at 1600 *g* 3 min, and placed at -53 °C for another 3 min. Then the *n*-hexane was removed and 200 µl of the aqueous solution was injected on the high-performance liquid chromatography system (HPLC pump model 420, Kontron Instruments, Milan, Italy with automatic injector 717 plus, Waters Assoc., Milford, MA). Quantitation was achieved by fluorescence detection of the eluate (fluorimeter RF 551, Shimadzu Corp., Kyoto, Japan). Experiments were performed in triplicate. Cellular levels were corrected for relative cell size, determined by the forward scatter of viable cells in FACS analysis using a FACStar flow cytometer (Becton Dickinson, Sunnyvale, CA).

Confocal Scanning Laser Microscopy

Single-cell suspensions of 5 x 10^5 cells/ml were allowed to attach overnight in 5 ml RPMI 1640 medium with 10% heat-inactivated FCS at 37 °C in a humidified atmosphere with 5% CO_2 on glass coverslips in petri dishes. GLC_4, GLC_4/ADR, A2780 and A2780AD cells were incubated for 15 min at 37 °C with equimolar doses of doxorubicin as control and MMRDX (1.5 and 15 µM), with or without 1 h 50 µM pre-incubation with the leukotriene (LTD_4) receptor antagonist MK 571 [13], to compare the cellular fluorescence. Viability of these cells after 0.5 and 1 h was checked by the trypan blue exclusion assay. After a quick rinse with Hanks' balanced salt solution without phenol red, coverslips were inverted, mounted on glass slides and kept on ice until analysis. Fluorescence of the cell suspensions was analyzed with a confocal scanning laser microscope (CSLM) (TCS Leica, Heidelberg, Germany) incorporating an inverted Leitz DMIRB microscope with a 100 x oil immersion lens. An argon/krypton laser was used for excitation at 488 nm and emission at 515 nm for doxorubicin and MMRDX. The intracellular distribution was qualitatively studied for 15-30 min per cell line. At least 30 cells of each cell line were studied.

Statistics

Cellular size and MMRDX levels in sensitive versus resistant cell lines were compared by unpaired Student's t-test. P values < 0.05 were considered significant.



■_____ = GLC_4, ■_ _ _ = GLC_4/ADR, ▾- - - - = A2780, ▾. . . . . = A2780AD

Figure 2 *Cell survival (%) ± SD (nM) in MTT assay after 4 days continuous MMRDX incubation (n=3)*

Results

Cytotoxicity of MMRDX

Survival curves of GLC_4, GLC_4/ADR, A2780 and A2780AD for MMRDX are shown in figure 2. A2780 was the most sensitive cell line for MMRDX while its doxorubicin resistant subline A2780AD was least sensitive with IC_{50} values (SD) of GLC_4 and GLC_4/ADR, respectively, 220 (10) and 400 nM (10), while IC_{50} values of A2780 and A2780AD were 180 (20) and 800 nM (80), respectively. As compared with their sensitive counterparts, cytotoxicity of MMRDX was only 1.8-fold reduced at the IC_{50} in the 300-fold doxorubicin resistant GLC_4/ADR cell line and 4.5-fold reduced in the 100-fold resistant A2780AD cell line. The viability of cells 0.5 and 1 h after incubation with 1.5 and 15 µM MMRDX and doxorubicin, determined by trypan blue exclusion, was more than 85% in all cell lines.

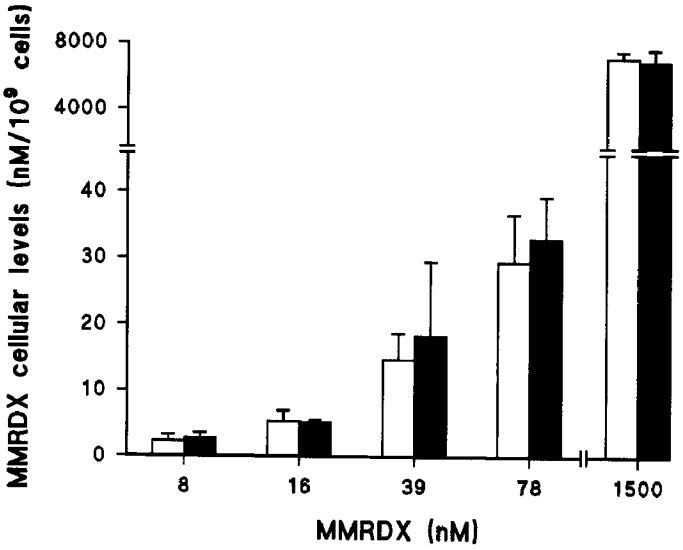
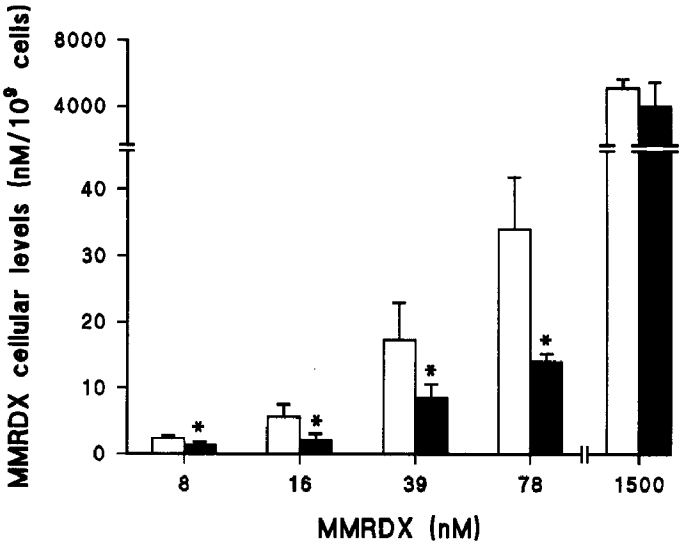


Figure 3A *MMRDX cellular levels ± SD (nM/10⁹ cells) after 1 h incubation with MMRDX in GLC₄ (open bars) and GLC₄/ADR (solid bars)*



* = statistically significant different MMRDX levels in resistant versus sensitive cell line ($p<0.05$)

Figure 3B *MMRDX cellular levels ± SD (nM/10⁹ cells) after 1 h incubation with MMRDX in A2780 (open bars) and A2780AD (solid bars)*

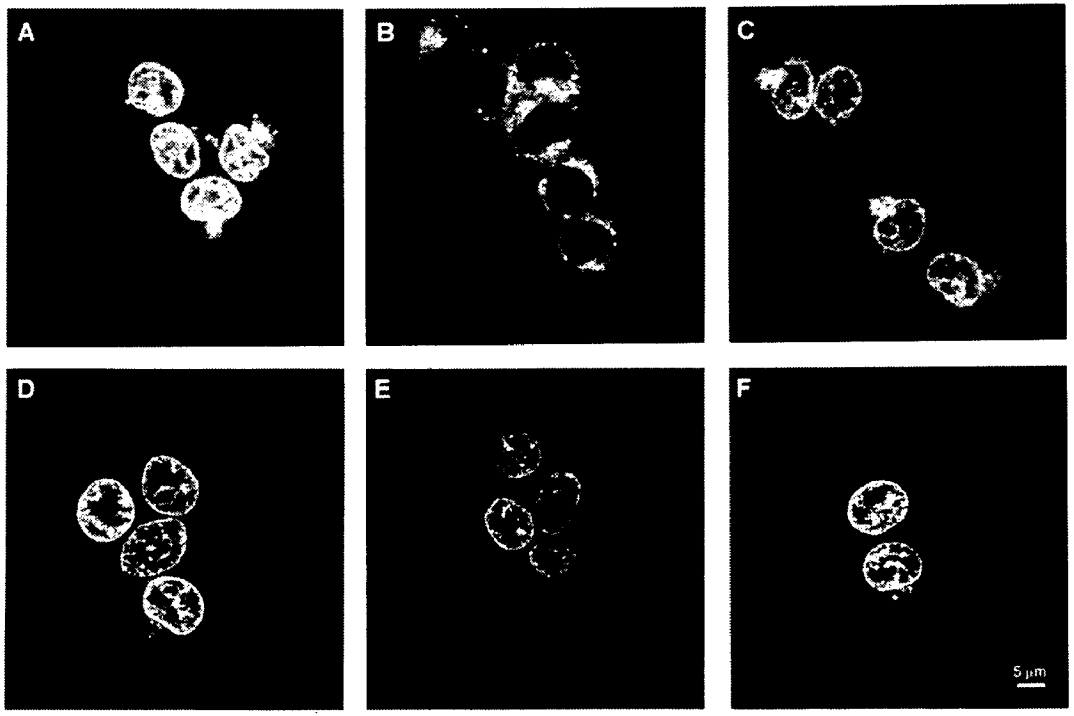


Figure 4 *Confocal scanning laser microscopy. A: representative cells after 15 min 15 µM doxorubicin incubation in GLC_4; B: idem in GLC_4/ADR; C: idem in GLC_4/ADR with MK 571 pre-incubation D: representative cells after 15 min 15 µM MMRDX incubation in GLC_4; E: idem in GLC_4/ADR; F: idem in GLC_4/ADR after MK 571 pre-incubation*

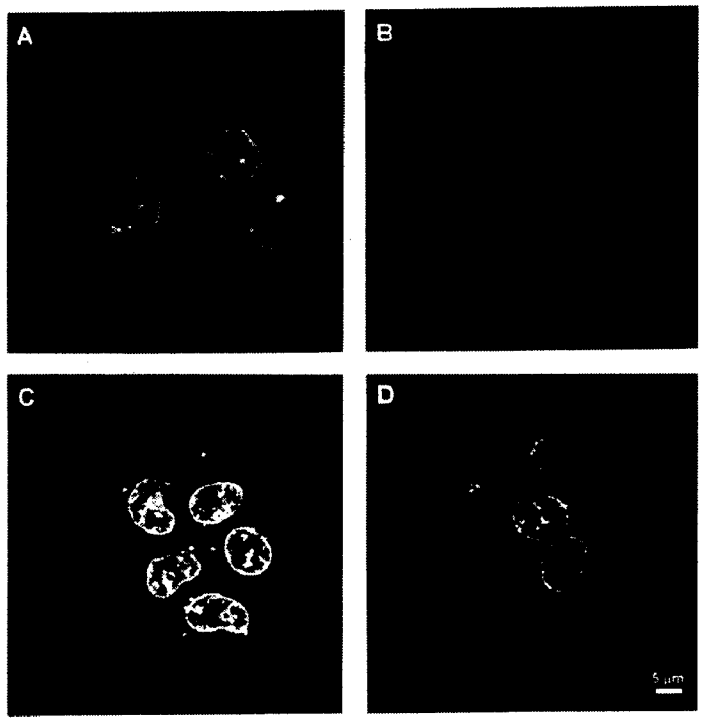


Figure 5 *Confocal scanning laser microscopy. A: representative cells after 15 min 15 µM doxorubicin incubation in A2780; B: idem in A7280AD; C: representative cells after 15 min 15 µM MMRDX incubation in A2780; D: idem in A2780AD*

Cellular MMRDX levels

Cellular MMRDX levels were equal in GLC_4 and GLC_4/ADR (figure 3A). In A2780AD cellular MMRDX levels were 2-fold lower compared to A2780 at the first 4 incubation steps (from lowest to highest concentration respectively $p < 0.01$, 0.0125, 0.025 and 0.0025) (figure 3B); at 1500 nM MMRDX drug levels in A2780 and A2780AD were not significantly different with respectively 5.1 and 4.0 $\mu M/10^9$ cells ($p > 0.05$). The 13-dihydro metabolite of MMRDX was less than 2% of the total MMRDX level in all cell lines at all incubation concentrations.

Confocal Scanning Laser Microscopy

To compare the distribution of doxorubicin and MMRDX in both doxorubicin sensitive and resistant cell lines, GLC_4 and GLC_4/ADR, and A2780 and A2780AD were incubated for 15 min with 15 µM doxorubicin and 1.5 and 15 µM MMRDX. Doxorubicin and MMRDX fluorescence were observed by the same laser power and instrument settings of the confocal microscope: Distribution of fluorescence after 15 min 15 µM doxorubicin incubation was only nuclear in the sensitive cells, while in the resistant cells doxorubicin fluorescence was cytoplasmic without any nuclear staining (figure 4A, B). In contrast, fluorescence after 15 min 15 µM MMRDX incubation was exclusively nuclear both in GLC_4 and in GLC_4/ADR cells (figure 4D, E). Incubation of GLC_4 and GLC_4/ADR cells with 1.5 µM MMRDX showed an identical staining pattern (data not shown). One hour 50 µM MK 571 preincubation restored the nuclear fluorescence pattern of doxorubicin in GLC_4/ADR (figure 4C), while the fluorescence pattern of MMRDX (15 µM) in GLC_4/ADR was unchanged after 50 µM MK 571 preincubation (figure 4F). Fluorescence detected after 15 µM doxorubicin incubation in A2780 and A2780AD cells showed a nuclear staining in A2780 cells, while doxorubicin fluorescence in A2780AD cells was exclusively cytoplasmic with strongly reduced intensity (figure 5A, B). Fluorescence detected in A2780 and A2780AD cells incubated for 15 min with 1.5 µM MMRDX revealed a nuclear staining in the sensitive line. In comparison, the resistant line showed cytoplasmic staining with reduced nuclear staining (data not shown). Fluorescence distribution after 15 min 15 µM MMRDX incubation was nuclear both in A2780 and A2780AD (figure 5C, D), although the staining in the resistant cells was less strong compared to the sensitive cells.

Discussion

This study describes cytotoxicity, cellular accumulation and intracellular distribution of MMRDX in a sensitive small cell lung cancer cell line and its resistant, MRP overexpressing subline, and a sensitive ovarian carcinoma cell line and its P-glycoprotein overexpressing resistant subline. MMRDX was able to overcome high levels of doxorubicin resistance in both types of doxorubicin resistant cell lines. Previously, Coley et al. have shown that MMRDX can overcome doxorubicin resistance caused by a decreased topoisomerase II activity [14]. Two important reasons for doxorubicin resistance in GLC_4/ ADR are reduced topoisomerase II levels compared to GLC_4, and overexpression of MRP, resulting in reduced doxorubicin levels in GLC_4/ADR compared to GLC_4. Topoisomerase I activity does not differ between the two GLC_4 cell lines. Cellular MMRDX levels observed in our study are in accordance with data from Grandi et al. who observed a high MMRDX accumulation in both sensitive and resistant cell lines expressing the MDR phenotype [15]. A previous study in GLC_4 and GLC_4/ADR showed 2.2-fold reduced cellular doxorubicin levels in GLC_4/ADR compared to GLC_4 [9]. In the present study MMRDX cellular concentrations were similar in these lines, suggesting that MMRDX is no substrate for MRP mediated cellular efflux. In the doxorubicin resistant P-glycoprotein overexpressing cell line A2780AD MMRDX levels were 2-fold reduced compared to A2780 at concentrations comparable to patients' peak plasma levels. MMRDX levels after 1.5 μM incubation in A2780 and A2780AD were the same, possibly due to a saturation of efflux pumps at this high drug concentration. Earlier results showed 2.9-fold reduced cellular levels in A2780AD compared to A2780 after incubation with 2 μM doxorubicin [10].

In P-glycoprotein and MRP overexpressing cell lines a discrepancy between levels of cross-resistance and cellular accumulation deficits has been observed for anthracyclines [1-3]. Additional data on intracellular distribution were sought for to deliver the bridging factor for the correlation between the resistance factor and intracellular anthracycline levels. A shift in concentration from the nucleus to the cytoplasm was observed in resistant cell lines, supporting the idea that anthracycline mediated cytotoxicity is effected mainly through their interaction with DNA. Recently, MRP was shown to be present in the cytoplasm and the Golgi region in tumor cells with high MRP overexpression in addition to its location at the cellular plasma membrane [16]. Doxorubicin transport was suggested for

intracellular MRP by the presence of intracellular secretory vesicles with MRP functioning as a transporter [4]. In this study we demonstrated that cellular MMRDX levels are not the sole predictors for cytotoxicity in the different cell lines. This observation is in accordance with previous studies with various morpholino and methoxypiperidinyl derivatives of daunorubicin in human colon carcinoma and leukemia cell lines. In these studies no correlation was observed between cellular accumulation and cytotoxicity [17, 18]. In the present study confocal scanning laser microscopy was used to determine subcellular distribution. The results obtained by confocal microscopy showed that MMRDX, in contrast to doxorubicin, is not prevented to reach the nucleus of the MRP overexpressing cells. In addition, in P-glycoprotein overexpressing cells the nuclear staining of MMRDX is less intense compared to the sensitive cells. These findings suggest that MMRDX is not a substrate for the MRP pump while P-glycoprotein might be able to prevent MMRDX from reaching its target, the nucleus.

Coley et al. observed unaffected cellular staining intensities after incubation with another morpholino anthracycline derivative, 3'-deamino-3'-(4-morpholino)adriamycin (MRDX, 1.5 μM), but reduced intensities for doxorubicin with a relative greater loss of nuclear fluorescence in a P-glycoprotein overexpressing mouse mammary tumor cell line [19]. They studied the large cell lung cancer cell line COR-L23/R overexpressing MRP which showed a highly intense area of perinuclear staining after doxorubicin incubation, absent in the P-glycoprotein overexpressing cell line, with a distribution suggestive of localization at the Golgi complex. The perinuclear Golgi-like staining remained, although markedly attenuated, after incubation with MRDX. As we observed no perinuclear fluorescence in the MRP overexpressing cell line GLC_4/ADR after incubation with 1.5 μM MMRDX, our data suggest that MMRDX with its methoxy group is better equipped than MRDX to overcome resistance due to a shift of the cytotoxic drug from the nucleus. Pre-incubation with the MRP blocker MK 571 before MMRDX incubation did not change the MMRDX fluorescence pattern in GLC_4 and GLC_4/ADR. However, we showed that the doxorubicin fluorescence shift from nucleus to cytoplasm could be prevented by pre-incubation with an MRP blocker.

Although the setting of this study does not allow a quantitative comparison of the fluorescence of the two anthracycline analogs, fluorescence observed after MMRDX incubation was far more intense than equimolar incubation

with doxorubicin. As the uptake of anthracyclines is dependent on their lipophilicity, these differences in fluorescence intensity between MMRDX and doxorubicin could at least partially be the result of the increased lipid solubility of MMRDX compared to doxorubicin. Additionally, quenching of fluorescence due to intercalation could result in underestimation of nuclear doxorubicin as the intercalation potency of methoxymorpholino doxorubicin is established at about 2-fold less than the intercalation potency of doxorubicin [2, 20, 5].

In conclusion, our cytotoxicity data show that the resistance factor of MMRDX in P-glycoprotein and MRP overexpressing cell lines is remarkably reduced compared to the resistance factor of doxorubicin. Cellular MMRDX levels and subcellular localization of MMRDX in P-glycoprotein and MRP positive cell lines suggest that MMRDX, in contrast to doxorubicin, is not transported by MRP. In vitro data justify ongoing research in the field of structurally modified anthracyclines and studies with these compounds in the clinic.

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